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Development of Methicillin Resistance in Clinical Isolates of *Staphylococcus sciuri* by Transcriptional Activation of the *mecA* Homologue Native to the Species

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The β-lactam resistance gene *mecA* was acquired by *Staphylococcus aureus* from an extraspecies source. The search for the possible origin of this gene has led to the identification of a close structural homologue of *mecA* as a native gene in the animal species *Staphylococcus sciuri*. Surprisingly, the overwhelming majority of *S. sciuri* isolates were fully susceptible to β-lactam antibiotics in spite of the ubiquitous presence of the *mecA* homologue in the bacteria. We now describe two unusual *S. sciuri* strains isolated from humans—SS-37 and SS-41—that showed resistance to methicillin associated with high rates of transcription of the *mecA* homologue and production of a protein resembling penicillin binding protein 2a, the gene product of *S. aureus mecA*. In strain SS-37 increased transcription of the *mecA* homologue was related to insertion of an IS*256* element upstream of the structural gene, and strain SS-41 had single nucleotide alterations in the promoter region of the *mecA* homologue which appear to be related to up-regulation of the rate of transcription. A third methicillin-resistant human isolate of *S. sciuri* that carries both the native *mecA* homologue and a methicillin-resistant *S. aureus* (MRSA) type *mecA*, strain K3, was now shown to be unstable in the absence of drug selection, causing the segregation of antibiotic-susceptible cells accompanied by the loss of the MRSA type *mecA*. These observations illustrate the remarkable variety of strategies available to bacteria for acquiring mechanisms of drug resistance in the in vivo environment.

The cellular mechanism of broad-spectrum β-lactam antibiotic resistance, by which *Staphylococcus aureus* and other staphylococcal species are capable of withstanding the inhibitory effects of all β-lactam antibiotics, including the semisynthetic ones such as methicillin, is based on acquisition of the *mecA* gene, the product of which is penicillin binding protein 2a (PBP2a), a protein with greatly reduced affinity to this class of antibiotics (10). The resistance mechanism conferred by *mecA* has been studied extensively not only because of the importance of resistant strains in the clinical setting but also because of the intriguing features of the mechanism itself, which still holds many unanswered questions. One such question is the origin of the *mecA* determinant, known for a long time to be exogenous to *S. aureus* and to other clinically relevant staphylococci (1, 5, 9).

In a search for the possible original source of *mecA*, we identified a genetic element closely related to the *S. aureus mecA* gene in the animal commensal species *Staphylococcus sciuri*, which is one of the most abundant staphylococcal species on our planet (12) and is only distantly related to *S. aureus* along taxonomic lines (21, 24). The broad range of *S. sciuri* biochemical activities, including the ability to use inorganic nitrogen salts as the sole source of nitrogen, is usually considered the reason for the wide range of host organisms and habitats of this bacterium, which include the skins of several animals as well as environmental reservoirs (6, 12). The *S. sciuri mecA* homologue has been found to be native to this species: it is carried by each of the 162 independent *S. sciuri* isolates tested to date, and these isolates were recovered over a wide range of time periods and from a variety of animal and human hosts (6, 7).

The *mecA* homologue identified in *S. sciuri* showed 79.5% DNA sequence similarity to the *mecA* gene of a methicillin-resistant *S. aureus* (MRSA) strain (27), indicating a close evolutionary relationship between these genetic determinants. The overall deduced amino acid sequence identity and similarity between the two elements were 79.98 and 87.69%, respectively. Most differences between the two sequences were found in the transglycosylase domain. When compared separately, the transpeptidase domains showed a similarity of 95.94% and an identity of 91.25%, while for the transglycosylase domains these values were 80.06 and 67.63%, respectively (27). Despite the similarities between the *S. sciuri mecA* and the MRSA *mecA*, the great majority of S. *sciuri* isolates showed no appreciable resistance to β-lactam antibiotics (6, 7). While the native *mecA* homologue was present in all *S. sciuri* isolates tested, a careful screen has identified a few *S. sciuri* isolates which also carried a second copy of *mecA* (6, 7). The two copies could be distinguished by cloning and sequencing: one was identified as *mecA1*, the homologue native to all *S. sciuri* strains, while the other, *mecA2*, was similar to the *mecA* gene found in methicillin-resistant strains of S. *aureus* (28).

Recently, Wu et al. (29) described a highly methicillin resistant step mutant obtained in the laboratory by antibiotic selection from the susceptible *S. sciuri* strain K1, which carries only *mecA1*, the homologue native to *S. sciuri*. Sequencing of *mecA1* from the resistant mutant K1M200 identified a single point mutation in the −10 consensus of the promoter region

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TABLE 1. Origins and relevant properties of *S. sciuri* strains used in this study

Strain	Origin	Methicillin MIC (μg/ml)[a]	PFGE pattern[b]	*mecA* type[c]	No. of IS*256* copies (kb)	Source or reference(s)
K3	Isolated from skin of a child in 1992 (neonatal ward, Mozambique)	6 (800)	X3	A1 + A2	2 (170, 265)	6, 12
K3y	New designation for K3 producing yellow (predominant) and white colonies	6 (800)	X2	A1 + A2	2 (170, 265)	This study
K3w	Variant of strain K3 producing only white colonies	3 (12.5)	X1	A1	2 (170, 265)	This study
K3wM200	Resistant laboratory mutant of strain K3w	200	X1	A1	4 (145, 170, 265, >480)	This study
SS-37	Isolated from the nasopharynx of a 2-year-old child attending a day care center in 1997	25 (800)	X1	A1	3 (145, 170, 265)	7
SS-41	Isolated from the nose of a 77-year-old patient in 1996 (hospital, Cape Verde)	3 (50)	Y	A1	0	7
K1 (control strain)	Isolated from the skin of a squirrel	3 (6)	W	A1	0	6, 29
K1M200 (control strain)	Resistant laboratory mutant of strain K1	200	W	A1	0	29

[a] Values in parentheses indicate the highest antibiotic concentration at which resistant subpopulations were found.
[b] PFGE patterns were reclassified in this work.
[c] A1, *S. sciuri* native *mecA;* A2, MRSA-like *mecA*.

which was associated with a striking increase in the level of transcription of the gene and the production of a protein that reacted with a monoclonal antibody against PBP2a, the product of the *S. aureus mecA* gene. Introduction of the up-regulated *mecA1* into an *S. aureus* strain with an inactivated *mecA* caused a significant increase in resistance to methicillin and the production of a PBP2a-like protein (29). These observations implied that *mecA1*, the gene homologue native to *S. sciuri*, has the capacity to confer resistance to methicillin and other β-lactam antibiotics provided that it acquires a more powerful promoter.

During a recent characterization of 28 *S. sciuri* strains isolated from humans, we found two strains that were particularly interesting in that they showed resistance to methicillin in spite of carrying the single copy of *mecA1* only (7). In the present work, we demonstrate that the *mecA* gene carried by these strains is indeed the native gene of *S. sciuri*, which is being constitutively expressed and producing a PBP2a-like protein. This is the first report of *S. sciuri* strains isolated in vivo, carrying only a copy of the native *mecA* gene, with the capacity to confer resistance to β-lactam antibiotics. Characterization of these and additional strains, submitted to in vitro step selection with antibiotic, allowed us to identify several distinct mechanisms by which the *S. sciuri mecA1* can be recruited by these bacteria to become an antibiotic resistance determinant.

MATERIALS AND METHODS

Bacterial strains. Properties of *S. sciuri* strains and constructs used in this study are summarized in Table 1. *Escherichia coli* strain MC1061-1 carried plasmid pMF13, which contains a 1.2-kb *Xba*I-*Pst*I fragment from the *mecA* gene of a MRSA strain cloned into plasmid pTZ19 (17). *S. sciuri* isolates were identified as gram-positive cocci which were catalase positive, did not produce coagulase, were able to ferment mannitol, were resistant to novobiocin, and were oxidase positive. Strain K3 was further studied by several other methods including ribotyping and DNA-DNA hybridization (12). Strains SS-37 and SS-41 were identified as *S. sciuri* with the ID32 STAPH kit (BioMerieux) (6). All strains were from the culture collections of the Molecular Genetics Laboratory of the Instituto de Tecnologia Química e Biológica (Oeiras, Portugal) and the Laboratory of Microbiology of The Rockefeller University (New York, N.Y.).

Media and growth conditions. *S. sciuri* strains were grown in tryptic soy broth (TSB) or agar (TSA) (Difco Laboratories, Detroit, Mich.) at 37°C. Luria-Bertani medium supplemented with 50 μg of ampicillin · ml^{-1} was used to grow the *E. coli* strain carrying the plasmid with the *mecA* probe.

For analysis of the different types of colonies produced by *S. sciuri* strain K3, the original culture was plated in TSA and incubated at 37°C for 48 h, followed by incubation at room temperature for 48 to 72 h. The number and color of the colonies were observed every 24 h. For the subsequent study of the different colonies, new cultures were prepared in TSB from distinct and well-isolated colonies.

Antibiotic step selection. For the step selection assay, a colony was picked from a TSA plate containing 3 μg of methicillin/ml and inoculated into TSB with the same concentration of the antibiotic. Upon growth, such cultures were used as inocula of a fresh TSB containing gradually increasing concentrations (in twofold steps) of methicillin. The existence of contaminants among the step-selected cultures was excluded by comparison of their pulsed-field gel electrophoresis (PFGE) patterns with those of the original strains. The *Cla*I-*mecA* polymorphisms of the selected cultures were analyzed for possible modifications that might have occurred in the *mec* region during the step selection procedure.

DNA methods. All routine DNA manipulations were performed by standard methods (2, 20). Restriction enzymes were purchased from New England Biolabs (Beverly, Mass.) and used as recommended by the manufacturer. Chromosomal DNAs for conventional gel electrophoresis and PFGE were prepared and run in conventional and PFGE gels as previously described (7).

Preparation of *mecA* DNA probes. The *mecA* probe used to hybridize both *Cla*I and *Sma*I chromosomal digests was prepared by restricting plasmid pMF13 with *Xba*I-*Pst*I and isolating the 1.2-kb fragment internal to *mecA* (17). The *mecA* probe used for Northern blot analysis, K1NP, is a 0.5-kb fragment, internal to the *mecA* of *S. sciuri* strain K1.

Preparation of the IS*256* DNA probe. The IS*256* probe was prepared by PCR amplification of a 1.14-kb fragment by using primers ICP17 (5′-CCCAGGAGG ACTTTTACATG) and ICP12 (5′-GCCTCACGCGCTAAGTTAAT).

Hybridization of chromosomal DNA digests with the *mecA* or IS*256* probe. Following restriction of the chromosomal DNA with *Cla*I or *Sma*I, the digestion fragments were resolved in gels (conventional or PFGE, respectively) and transferred to nylon membranes by vacuum transfer. Membranes containing the digested DNAs were hybridized with the *mecA* or IS*256* probe under high-stringency conditions using the ECL Random Prime nonradioactive labeling system (RPN 3030; Amersham, Little Chalfont, Buckinghamshire, United Kingdom) according to the manufacturer's recommendations.

PCRs. PCR amplification of DNA for sequencing or probe preparation was performed with *TTh* polymerase (HT Biotechnology Ltd., Cambridge, United Kingdom) on a Perkin-Elmer Applied Biosystems (Cheshire, United Kingdom) Gene Amp PCR System 9600. When necessary, PCR products were purified with the Wizard PCR Preps DNA purification system (Promega, Madison, Wis.).

Determination of DNA sequence. DNA sequencing was performed with automated DNA sequencing systems at the sequencing facilities located at the Gulbenkian Institute (Oeiras, Portugal) and The Rockefeller University. Nucleotide and amino acid sequences were analyzed with DNAStar software (Lasergene, Madison, Wis.).

RNA preparation and Northern blot analysis. RNA was prepared with the FastRNA isolation kit (Bio 101, Vista, Calif.) according to the recommendations of the manufacturer. RNA samples (5 μg each) were resolved in a 1.2% agarose–0.66 M formaldehyde gel in MOPS (morpholinepropanesulfonic acid) running buffer (Sigma Chemical Company, St. Louis, Mo.), with RNA size markers

(Promega). RNAs were blotted onto a nitrocellulose membrane with the Turboblotter alkaline transfer system (Schleicher & Schuell, Keene, N.H.). Probe labeling with [α-^{32}P]dCTP was carried out with the Megaprime System (Amersham Life Science Inc., Arlington Heights, Ill.), and hybridization took place under high-stringency conditions.

Primer extension analysis. The 5′ ends of transcripts of *S. sciuri mecA* were determined by primer extension as previously described (3, 29). Briefly, the MAK1PE oligonucleotide, 5′-TTCAATGGCATCAATTGTTTCG (29), was end labeled with [γ-^{32}P]dATP by using a 5′-end labeling kit (Amersham Life Science Inc.) and was hybridized with 25 μg of each RNA preparation. Reverse transcription was carried out with SuperScript RT (Gibco-BRL, Gaithersburg, Md.). After heat inactivation of the enzyme, the reaction product was incubated with RNase H (Roche Diagnostics GmbH, Mannheim, Germany), purified by extraction with phenol-chloroform-isoamyl alcohol, and ethanol precipitated. Analysis of the extended products was carried out on 7.5% polyacrylamide-urea gels. Sequencing reaction mixtures containing K1 DNA primed by oligonucleotide MAK1PE were carried out with the Sequenase system (U.S. Biochemical Corp., Cleveland, Ohio) and applied in parallel lanes on the gel.

Membrane purification. Membrane proteins were prepared from bacterial cultures as previously described (23). Proteins were quantified by using the Dc Protein Assay kit (Bio-Rad Laboratories, Hercules, Calif.).

Western blotting. For detection of PBP2a in the membrane protein fraction, 30 μg of each membrane protein preparation was resolved on 8% acrylamide gels and transferred to nitrocellulose membranes by Western blotting as previously described (29). Incubation with a monoclonal antibody against PBP2a of a MRSA strain (Eli Lilly & Co., Indianapolis, Ind.) was carried out with the ECL Western blot analysis system (Amersham Pharmacia Biotech) (29).

RESULTS

Characterization of methicillin-resistant isolates of *S. sciuri*. The *S. sciuri* strain K3, originally recovered from the skin of a child in a pediatric ward of a hospital in Mozambique, was already known to be unusual in several respects. (i) In contrast to most *S. sciuri* animal isolates, which are susceptible to β-lactam antibiotics, strain K3 exhibited heterogeneous resistance to methicillin (6). In cultures of strain K3 the methicillin MIC was 6 μg/ml for the majority of cells, but cells capable of growing in the presence of high concentrations of methicillin (up to 800 μg/ml) were also present at frequencies of 10^{-5} to 10^{-7}. (ii) *S. sciuri* strain K3 was shown to carry two *mecA* sequences: *mecA1*, corresponding to the *mecA* gene native to all *S. sciuri* strains, and *mecA2*, indistinguishable from the resistance gene *mecA* typical of MRSA isolates (6, 28).

During repeated plating and restreaking of colonies, we noted yet another unusual property of this strain. Plating strain K3 on TSA, we observed that the predominantly yellow colonies were occasionally mixed with white colonies (Fig. 1). We followed up this observation by plating cultures of the original strain K3 and also cultures grown from two distinct colonies picked from the TSA plate: a yellow colony (K3y) and a white colony (K3w). The three cultures were plated on TSA at various dilutions, and the numbers of yellow and white colonies were counted after 48 h of incubation at 37°C. Table 2 shows the results of two such experiments. The results indicate that the yellow colonies kept segregating both yellow and white colonies, although the yellow ones predominated. On the other hand, the white colonies produced only white colonies. Following additional incubation at room temperature for 2 to 3 days, white sectors of growth appeared in some of the yellow colonies (Fig. 1). Determination of the antibiotic resistance of colony variants K3y and K3w by population analysis showed that K3y was resistant to methicillin and had retained the heterogeneous population structure of the original strain K3, while in cultures of K3w the majority of cells had lost resistance (Table 1).

The *Sma*I PFGE profiles of chromosomal DNAs from pure cultures of the yellow (K3y) and white (K3w) colony variants showed differences in three bands: two fragments of approximately 160 and 30 kb present in the yellow variant were absent from the white segregant, which instead had a new band of 120 kb. The remaining *Sma*I fragments were identical (Fig. 2A). Testing with the *mecA* DNA probe identified two hybridizing bands, of 160 and 145 kb, in strain K3y and a single hybridizing band of 145 kb in strain K3w (Fig. 2B). The change of one *Sma*I band from 160 kb in K3y to 120 kb in K3w, corresponding to the loss of approximately 40 kb in the chromosome of the white culture, was accompanied by the loss of the 160-kb *mecA*-hybridizing band carried by the original strain K3 (Fig. 2B). Hybridization of chromosomal *Cla*I digests with the same *mecA* probe confirmed that during segregation of the white variant one of the *mecA* copies was lost and also demonstrated that the copy lost was the *S. aureus* type *mecA2*. This conclusion was based on the fact that the *S. aureus* type *mecA2* comprises a *Cla*I restriction site producing two *mecA*-hybridizing fragments after *Cla*I digestion, while the *S. sciuri mecA1* has no recognition site for *Cla*I (27). While strain K3y produced three *Cla*I-*mecA* fragments (one from *mecA1* and two from *mecA2*) of 13.5, 5.4, and 2.2 kb, strain K3w showed a single *Cla*I-*mecA* fragment, of 13.5 kb (Fig. 2C), which corresponded to the *mecA1* copy. The nature of the 30-kb band, which was also lost during segregation of the K3 white variants, is unknown.

Five *S. sciuri* strains were selected for more detailed characterization: strain K3y (new designation for strain K3 producing predominantly yellow colonies); strain K3w, the nonpigmented (white) segregant of K3; K3wM200, a highly methicillin resistant mutant of K3w obtained in the laboratory by step selection; and two naturally occurring methicillin-resistant *S. sciuri* strains, SS-37 and SS-41, each of which carried only the gene homologue *mecA1* native to *S. sciuri*. Strains SS-37 and SS-41 expressed heterogeneous resistance to methicillin: methicillin MICs for the majority of cells in cultures of SS-37 and SS-41 were 25 and 3 μg/ml, respectively, and subpopulations of cells capable of growing at 800 (SS-37) or 25 (SS-41) μg of methicillin/ml were present in cultures of these strains (7) (Table 1).

PFGE analysis of chromosomal *Sma*I digests was used to test the relatedness of the five S. *sciuri* strains studied. As already described, strains K3y and K3w and the original strain, K3, showed closely related PFGE patterns (Fig. 2A), shared by the resistant laboratory derivative K3wM200 (data not shown). Unexpectedly, the same PFGE pattern was also shared by strain SS-37. The similarity of PFGE patterns was surprising in view of the different origins of these strains: SS-37 was isolated from the nasopharynx of a child in 1997 (7), and strain K3 was recovered from skin at a neonatal ward of a hospital in East Africa in 1992 (6, 12). Strain SS-41, recovered from the nares of an elderly patient in Cape Verde in 1996 (7), had a completely different PFGE pattern.

Sequence analysis of *mecA* and upstream region. The parallel losses of methicillin resistance and the second, *S. aureus* type copy of *mecA* during segregation of white K3w colonies indicates that the methicillin-resistant phenotype of K3y was

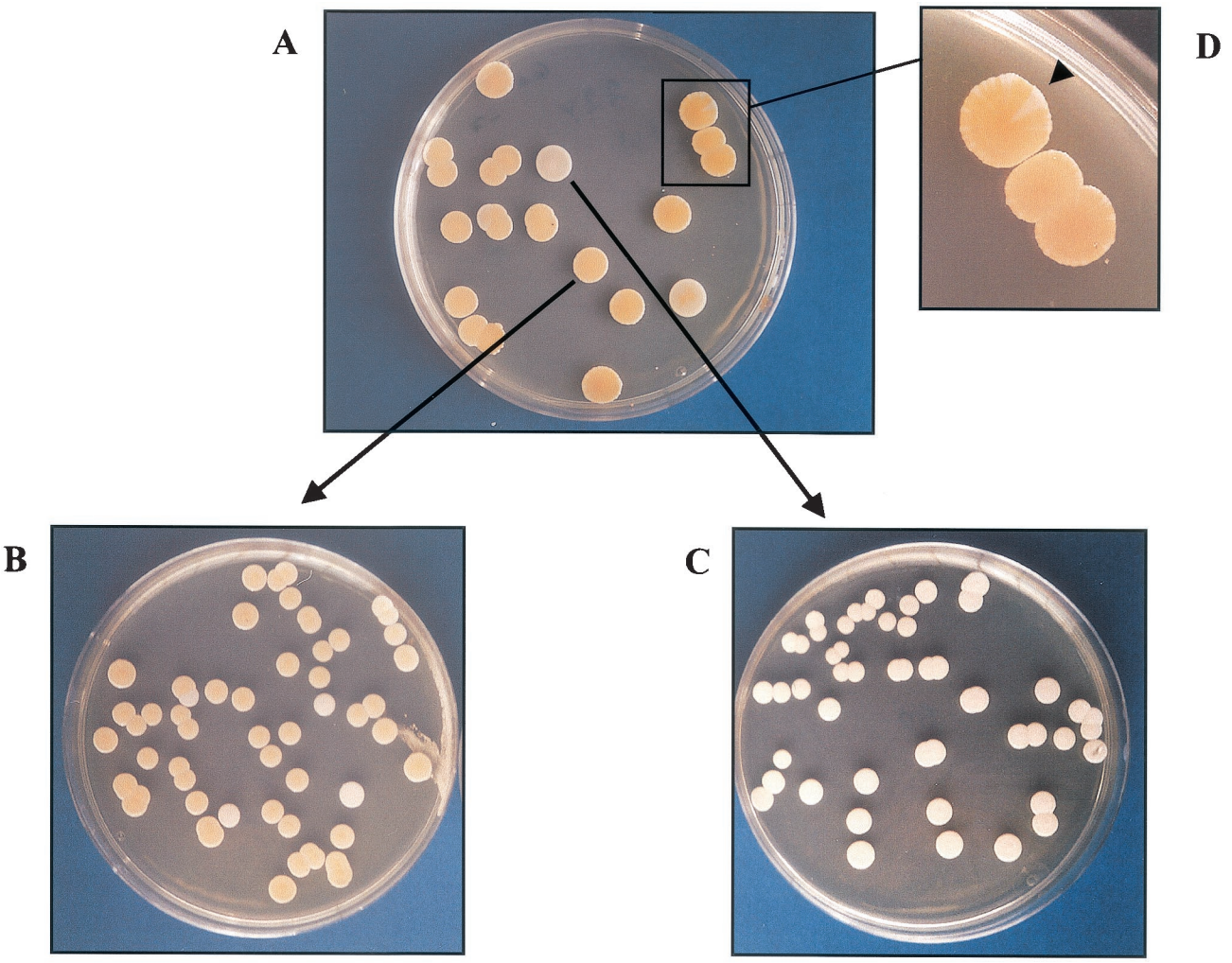

FIG. 1. Segregation of K3 yellow and white variants in TSA after incubation at 37°C for 48 h. (A through C) Plate prepared from a K3 culture showing yellow and white colonies (A), which generate, respectively, both yellow and white colonies (B) or only white colonies (C). (D) Yellow colonies from a K3 culture showing white sectors of growth (arrowhead).

associated with the presence of *mecA2* in this strain. In order to determine the basis for the methicillin resistance in the other three strains studied, we sequenced the *mecA* gene and the region upstream in K3wM200 and in strains SS-37 and SS-41. Each of these strains carried a sequence virtually identical to that of *mecA1*, identified earlier as the *mecA* homologue native to all *S. sciuri* isolates (27, 28). Minor deviations from this common sequence were a change in 2 bp within the *mecA*-coding region (551 to 552 bp) in strain SS-37, leading to the replacement of an isoleucine by a valine residue, and the replacement of a thymine by a guanine residue at position 1474 of the coding sequence in both strains SS-37 and SS-41, leading to the replacement of a tyrosine by aspartate. The presence of an aspartate residue in this position was previously described for *S. sciuri* type strain K1 (27). For each of the three strains studied, a well-conserved ribosome-binding site (GGGAGG) was detected, 6 bp upstream of the putative *mecA* initiation codon.

Examination of the DNA sequence further upstream of *mecA* revealed that in both SS-37 and K3wM200, an additional 1.3-kb fragment was inserted in the promoter region of *mecA*. This element was identified as a complete copy of IS*256* that inserted in slightly different positions in the two genomes: at 109 and 92 bp upstream of the start codons of SS-37 and K3wM200 *mecA*, respectively. In both cases, the insertion element was oriented in the same transcriptional direction as the

TABLE 2. Segregation of yellow and white colonies from *S. sciuri* strain K3 after 48 h of incubation at 37°C

Culture[a]	No. of colonies (CFU per ml) in the:			
	1st assay		2nd assay	
	Yellow	White	Yellow	White
K3 (original)	3.8×10^8	1.2×10^8	2.9×10^8	8.9×10^7
K3y	5.2×10^8	7.5×10^6	3.0×10^8	5.0×10^5
K3w	0	3.3×10^8	0	3.2×10^8

[a] K3y, culture prepared from a yellow colony; K3w, culture prepared from a white colony.

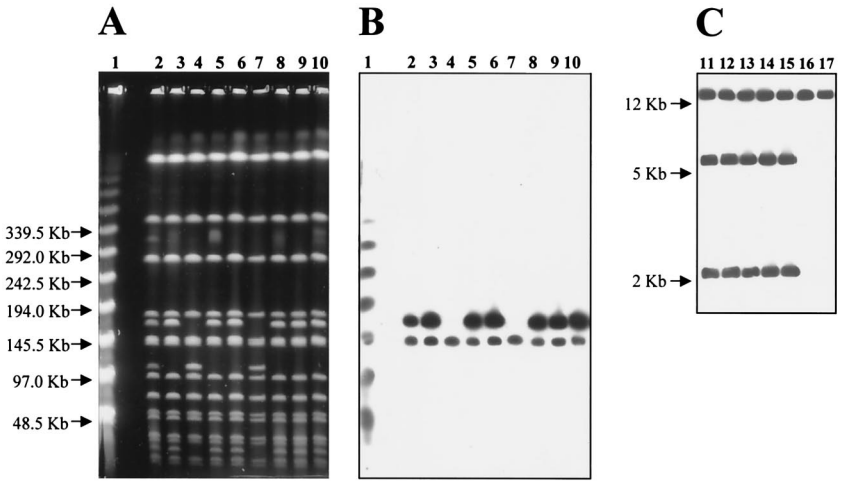

FIG. 2. Genotypic analysis of the K3y and K3w variants. (A) PFGE patterns of the K3y and K3w variants after *Sma*I digestion. Lane 1, PFGE lambda marker; lanes 2 to 4, *Sma*I restriction patterns of K3 (original culture), K3y, and K3w, respectively; lanes 5 to 10, *Sma*I restriction patterns of cultures prepared from independent yellow (lanes 5, 6, and 8 to 10) or white (lane 7) segregated colonies. (B) Hybridization of the K3y and K3w variant *Sma*I digests shown in panel A with a DNA probe for *mecA*. (C) Hybridization of the K3y and K3w variant *Cla*I digests with the DNA probe for *mecA*. Lanes 11 to 17, *Cla*I restriction patterns of cultures prepared from independent yellow (lanes 11 to 15) or white (lanes 16 and 17) segregated colonies. Arrows indicate molecular size marker positions.

mecA gene, and it was flanked by an 8-bp duplication: TGAT TTTA for SS-37 and TTTTAATT for K3wM200.

No IS*256* was detected in the third methicillin-resistant strain, SS-41. Examination of 618 bp of DNA sequence upstream of the SS-41 *mecA* revealed only three differences in comparison to K3 *mecA1*: a replacement of cytosine by thymine at 143 bp upstream of the start codon and two additional single-base differences further upstream at 449 and 464 bp.

Transcriptional analysis of *mecA*. A DNA probe internal to the *S. sciuri mecA1* was used to analyze transcription of this gene by Northern blot analysis. Single transcriptional units were detected in the methicillin-resistant strains SS-37, SS-41, and K3wM200, while strain K3w (and strain K1, used as a negative control) showed no hybridization signal under the conditions used. The transcripts identified for strains SS-37, SS-41, and K3wM200 varied in size and intensity of the hybridization signal (Fig. 3). Strain K3wM200 showed partial RNA degradation in the Northern blot assay, probably resulting from the relative instability of the transcript in this laboratory-selected mutant.

In order to determine if these strains shared the same transcriptional start site, we mapped the 5′ ends of all transcripts by primer extension. As a control, we used the pair of *S. sciuri* strains K1 and K1M200; a point mutation in the −10 region of the step-selected mutant K1M200 was associated with increased transcription of *mecA* (29). K1 DNA was used as a sequencing ladder to determine the sizes of the extended products.

Strains SS-37, K3wM200, and SS-41 all showed different transcriptional starts, which also differed from the one previously determined for K1M200 (29). The transcription starts for strains SS-37 and K3wM200 were mapped 80 and 64 bp upstream of the *mecA* start codon, respectively, close to the 3′ end of IS*256* (Fig. 4). Based on the +1 sites determined for SS-37 and K3wM200, the −35 promoter region should correspond to the outward-facing 3′ end of IS*256* (nucleotides TT GACT) as already described for *S. aureus* (15). The −10 region for strains SS-37 and K3wM200 would correspond to nucleotides TTTAAT and TATTAT, respectively (Fig. 4). The transcriptional initiation site of strain SS-41 was located 113 bp upstream of the *mecA* start codon. Based on the +1 site identified for SS-41, the consensus −10 and −35 regions would be, respectively, TATAGT and TTCCTT. The −35 region would include the cytosine-thymine substitution previously identified for this strain (see Fig. 4).

Western blot analysis. Expression of the *mecA* gene homologue carried by strains SS-37, SS-41, K3w, and K3wM200 was tested in membrane protein preparations with a monoclonal antibody against PBP2a. Membrane preparations of strains K1 and K1M200 were used as negative and positive controls, respectively (29). All three resistant strains, SS-37, SS-41, and K3wM200, produced a protein that reacted with the antibody for PBP2a, with the same molecular mass as the protein produced by K1M200, i.e., 84 kDa (Fig. 5). (29). The amounts of protein produced by strains SS-37, SS-41, and K3wM200 were greater than the amount produced by strain K1M200. The

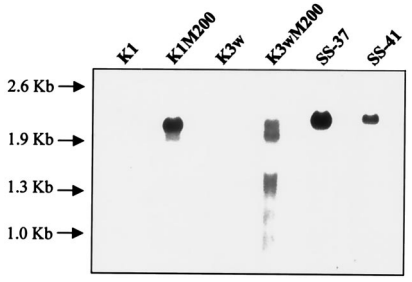

FIG. 3. Northern blot analysis of *mecA* transcription in *S. sciuri* strains isolated in vivo (K1, K3w, SS-37, and SS-41) or methicillin step selected (K1M200 and K3wM200).

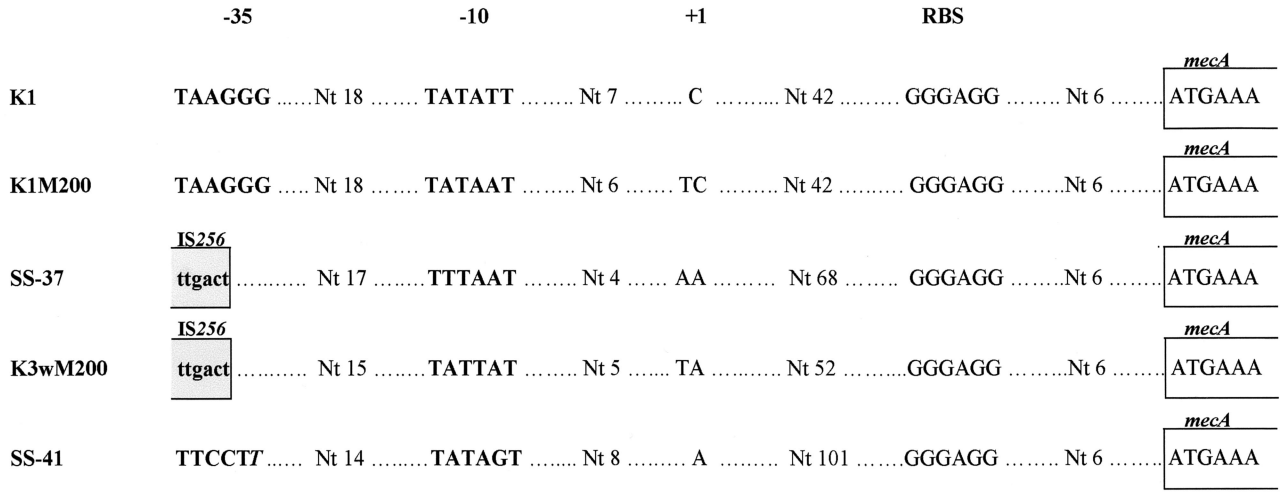

FIG. 4. Nucleotide sequence analysis of the region upstream of *mecA* for strains K1, K1M200, SS-37, SS-41, and K3wM200, specifying the mapped 5′ ends of the mRNAs identified by primer extension (+1), the putative promoter regions (−10 and −35), and the putative ribosome-binding sites (RBS). The putative promoter regions are boldfaced. The point mutation identified in SS-41 is italicized. White and gray open boxes represent the 5′ end of *mecA* and the 3′ end of IS*256*, respectively. The 3′ region of IS*256* is lowercased.

methicillin-susceptible strains K1 and K3w showed no detectable production of protein reacting with the antibody (Fig. 5).

IS*256* hybridization patterns. The presence of IS*256* in the chromosomes of the *S. sciuri* strains was analyzed by hybridizing *Sma*I digests with a DNA probe for this insertion sequence. Since IS*256* does not contain any restriction site for *Sma*I (4), we concluded that the number of hybridizing bands represented the minimum number of copies of IS*256* in the chromosome. Strain SS-37 showed three hybridizing *Sma*I bands of approximately 145, 170, and 265 kb. Strains K3y and K3w hybridized with the IS*256* probe in two *Sma*I fragments of 170 and 265 kb. In strain K3wM200 two additional DNA fragments of approximately 145 and >480 kb—i.e., a total of four *Sma*I bands—hybridized with this probe (Fig. 6). Most interestingly, the 145-kb *Sma*I fragment in which one of these additional IS*256* copies was found was the one carrying the *mecA* gene (see Fig. 2).

The additional observation that the size of the single *Cla*I-*mecA* hybridization band was reduced from 13.5 kb in the parental strain K3w to 8 kb in K3wM200 (data not shown) is consistent with the insertion of one IS*256* copy upstream of *mecA* during the step selection procedure, since this insertion sequence contains a *Cla*I recognition sequence (4).

The putative number of IS*256* copies carried by each strain was confirmed by hybridizing chromosomal *Eco*RI digests with a probe for IS*256*, which does not contain *Eco*RI restriction sites (data not shown).

DISCUSSION

The findings described in this paper provide interesting insights into bacterial adaptation to antibiotic pressure in the in vivo environment. They also allow comparison of the similarities and contrasts in the mechanisms of antibiotic resistance evolving in vivo and in vitro in a staphylococcal species that is relatively rare in the human sphere. *S. sciuri* is a natural commensal and skin inhabitant of rodents and primitive mammals, but occasionally *S. sciuri* strains have been isolated from human sources as well (7, 11, 12, 13, 16, 25, 26).

We studied three *S. sciuri* isolates recovered from humans (one from colonization and two from clinical specimens) that showed a significant degree of methicillin resistance. This phenotype represents a deviation from the typical susceptibility profile of the *S. sciuri* species, most members of which are completely susceptible to β-lactam antibiotics, including methicillin (6). The observations described in this paper suggest that each of these three methicillin-resistant natural isolates of *S. sciuri* has followed distinct strategies to adapt to the pressure of antimicrobial agents in the human-clinical environment.

The *mecA* region of the first of these drug-resistant strains, strain SS-41, was compared to the already characterized *mecA1* region of strain K3. Differences within a 618-bp region sequenced upstream of the *mecA* of strain SS-41 were minor: one single-base change each at positions 449 and 464, and a change of cytosine to thymine at 143 bp upstream of the start codon. The transcription of *mecA* in strain K3w was very poor in contrast to the strong transcription of the gene in strain SS-41 (Fig. 3). We suggest that the striking increase in the transcription rate (and the appearance of the drug-resistant phenotype)

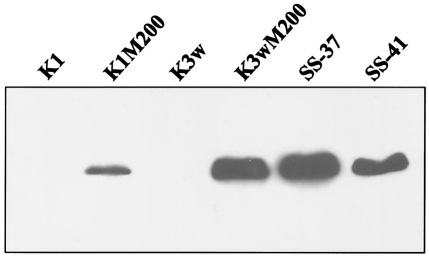

FIG. 5. Western blot analysis of membrane preparations against an antibody for PBP2a. Strains SS-37, SS-41, and K3wM200 produced a protein that reacted with the antibody for PBP2a, with the same molecular mass as the protein produced by K1M200, i.e., 84 kDa (29).

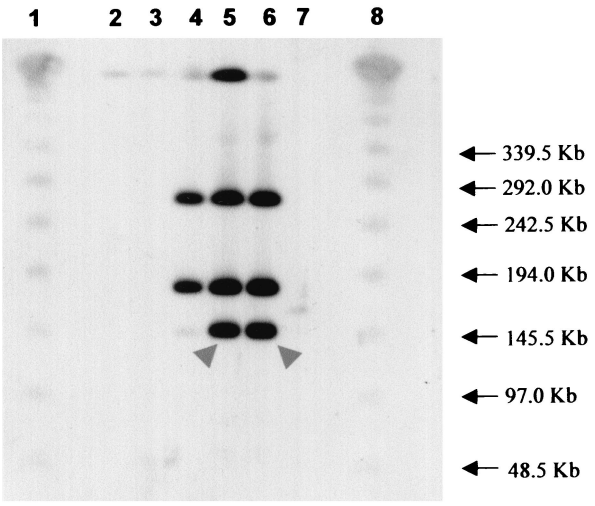

FIG. 6. Hybridization of *Sma*I digests with a probe for IS*256*. Lanes 1 and 8, PFGE lambda ladder; lanes 2 to 7, K1, K1M200, K3w, K3wM200, SS-37, and SS-41, respectively. Arrowheads indicate the *Sma*I bands hybridizing with *S. sciuri mecA* for strains K3wM200 and SS-37.

in SS-41 is related to the change from cytosine to thymine upstream of *mecA*. This interpretation is based on the overall similarity of the *mecA* upstream regions in strains K3w and SS-41 and on the primer extension data positioning the transcription start site close to the thymine residue at position 143. Testing this hypothesis would require cloning and expression of SS-41 *mecA* DNA in a methicillin-susceptible strain. The methicillin resistance mechanism proposed for strain SS-41 is analogous to the mechanism recently identified in a laboratory mutant of *S. sciuri*, in which increased transcription of the *mecA* homologue and appearance of drug resistance were caused by a single-base alteration in the −10 region of the promoter (29). The fact that SS-41 was isolated from a clinical source suggests that this survival strategy may also be used by strains exposed to antibiotic pressure in vivo.

In the second heterogeneously methicillin resistant *S. sciuri* strain, SS-37, the native *mecA1* gene appears to be mobilized to become a potential antibiotic resistance gene by up-regulation of its transcription, resulting in the appearance of large quantities of a PBP2a-like protein in the cell extract. Sequencing of *mecA* in this strain revealed that the upstream region contained an IS*256* element. The formation of highly active hybrid promoters by IS*256* has been previously described in *S. aureus* (15). The transcriptional data obtained indicate that insertion of IS*256* in the upstream region of SS-37 *mecA* has indeed created such a hybrid promoter, which was hijacked by the *mecA* homologue as a surrogate promoter. The presence of IS*256* in two of the methicillin-resistant natural isolates was surprising, since this insertion sequence has not been detected before in *S. sciuri* isolates and our own data indicate that it is rarely carried by this species (I. Couto, unpublished data). Therefore, it seems reasonable to hypothesize that *S. sciuri* strains carrying IS*256*, in particular strain SS-37, which carries several copies of IS*256*, must have acquired this insertion sequence from an exogenous source, possibly from some human species of staphylococci (8, 14) or other bacteria (18, 19) known to carry multiple copies of IS*256*.

The third strain analyzed in this study, strain K3, had already been shown to carry two *mecA* genes: *mecA1*, typical of all *S. sciuri* isolates, and a second copy, *mecA2*, indistinguishable from the *S. aureus mecA* determinant (28). The new observations described in this paper demonstrate that strain K3 is unstable: it segregates methicillin-susceptible nonpigmented (white) and methicillin-resistant yellow colonies. The yellow colonies continue to segregate yellow and white colonies, while the white colonies breed white progeny only. Our observations further show that the loss of antibiotic resistance in the white colonies was associated with loss of the *S. aureus* type *mecA2* gene from these segregants, indicating that the source of methicillin resistance in strain K3 was *mecA2*.

Additional revealing observations were made by further studying K3w, the nonpigmented, methicillin-susceptible segregant of strain K3 from which the *S. aureus* type *mecA2* was lost. We were interested in determining what type of drug resistance mechanism—if any—such methicillin-susceptible segregants could generate during antibiotic selection in vitro. Exposure of K3w to gradually increasing concentrations of methicillin rapidly yielded the laboratory mutant K3wM200, a strain for which the original methicillin MIC of 3 μg/ml characteristic of strain K3w was increased to 200 μg/ml. Examination of this step mutant for the transcription rate of the *S. sciuri mecA* homologue showed a striking increase over that of the parental strain K3w. Cell extracts from K3wM200 contained a protein that reacted with a monoclonal antibody prepared against PBP2a, the *S. aureus mecA* gene product.

Both the *Sma*I-IS*256* and the *Cla*I-IS*256* hybridization results suggest that during the step pressure, K3wM200 has recruited one of the IS*256* elements carried by K3w—presumably through an intrachromosomal translocation or duplication—such that this insertion sequence could assume the role of a surrogate promoter for increased expression of the *mecA* homologue, as described for strain SS-37. Autonomous movement of IS*256* and multiple independent insertions of this element into the staphylococcal chromosome have been observed (8, 14). Ziebuhr et al. (30) have shown that phase variation in biofilm production by *Staphylococcus epidermidis* was caused by alternating insertion and excision of IS*256* at different sites of the *ica* gene cluster; other authors have suggested that IS*256* transposition into the *llm* gene is involved in methicillin heteroresistance in *S. aureus* (15). Our own results suggest that IS*256* may also play a role in the modulation of methicillin resistance in *S. sciuri*.

Putting all observations described in this paper together allows one to suggest that strains SS-37, K3w, and K3 are related to one another along a putative evolutionary sequence of events, summarized in Fig. 7. We propose that the ancestral strain in this sequence was a methicillin-susceptible *S. sciuri* strain with the genotype of K3w or SS-37 (PFGE pattern X1 in Table 1) carrying solely the native *mecA* homologue. This ancestral strain drifted over into the human-clinical environment, where it acquired the IS*256* element. We propose that this strain (the hypothetical SS-37* in Fig. 7), equipped with several copies of IS*256*, was in fact the direct precursor of strain SS-37, which under exposure to some β-lactam antibiotic in the environment recruited one of the acquired IS*256* ele-

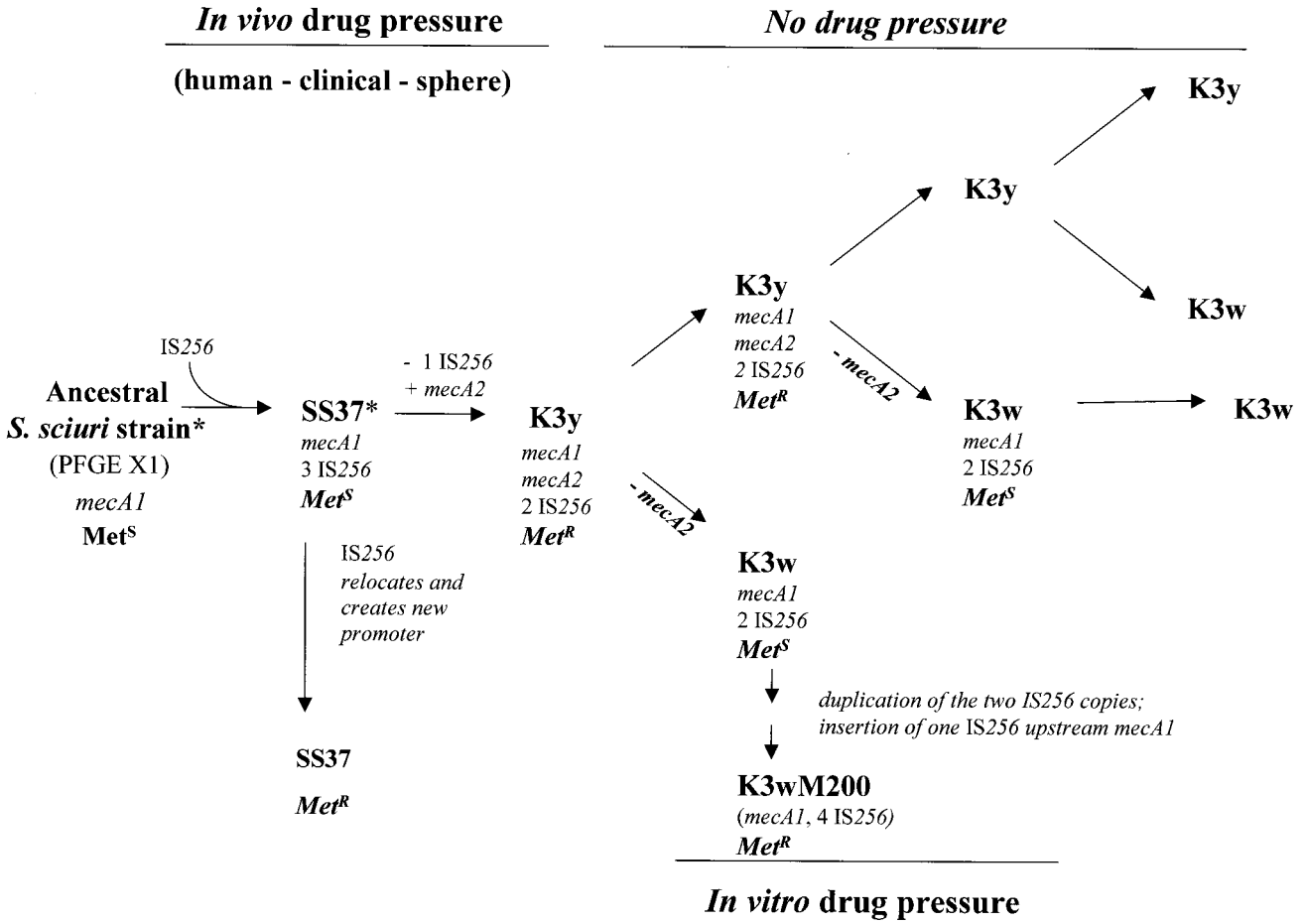

FIG. 7. Putative evolutionary sequence of events for strains K3w, K3y, and SS-37.

ments as a promoter, allowing overexpression of the *mecA* homologue and consequent expression of methicillin resistance. A second lineage of SS-37* did not recruit the acquired IS*256* sequence for resistance but rather evolved another resistance strategy, which involved acquisition of a complete copy of the *mecA2* gene from a MRSA or methicillin-resistant coagulase-negative staphylococcus strain, and thus emerged as strain K3 or K3y, equipped with two copies of *mecA*—one from MRSA and one from the *S. sciuri* native *mecA*. Strain K3y is able to cope with antibiotic pressure, as shown by its heterogeneous resistance profile. However, in the absence of antibiotic pressure, this strain is unstable and segregates white colonies which are antibiotic susceptible and from which the *S. aureus* copy of *mecA2* is lost; by this process K3w, which carries only the *S. sciuri* copy of *mecA1* plus the IS*256* sequences, is generated. When such a strain is exposed to antibiotic pressure in the laboratory environment, the bacterium again uses the first strategy for resistance: the hijacking of the IS*256* element to provide a strong promoter driving the overexpression of the *mecA* gene, resulting in the methicillin-resistant phenotype of strain K3wM200. The reversibility of the K3y/K3w system may be considered a model to illustrate an early stage in the evolution of a drug resistance mechanism in which maintenance requires continuous drug selection; otherwise, the mechanism is lost. The implication is that at this reversible stage of evolution, compensatory ("fitness") mutations have not yet stabilized the resistance mechanism, and the biological price of maintaining it in a nonselective environment is not worth its "cost."

The segregation of white K3 colonies resembles the spontaneous formation of nonpigmented or sectored colonies in *S. aureus* cultures reported earlier by Servin-Massieu (22). The mechanism of cosegregation of pigmentation with methicillin susceptibility observed for this strain is not clear. The loss of the yellow color together with the loss of the second, *S. aureus* type copy of *mecA* suggests that genetic determinants of pigmentation may be located in the same 40-kb DNA which is deleted from the chromosome of strain K3w.

The observations described in this report provide further evidence suggesting that the *mecA* homologue ubiquitous in the animal commensal species *S. sciuri* may very well have been the evolutionary precursor of the *mecA* determinant in MRSA. In this view the assembly of the *mec* element, i.e., the large 30- to 60-kb "foreign" DNA in which the *mecA* determinant of *S. aureus* is embedded, may have occurred in a parallel, or possibly a later, evolutionary sequence of events.

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REFERENCES

1. **Archer, G. L., and D. M. Niemeyer.** 1994. Origin and evolution of DNA associated with resistance to methicillin in staphylococci. Trends Microbiol. **2:**343–347.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1992. Short protocols in molecular biology. Greene Publishing Associates and John Wiley & Sons, Inc., New York, N.Y.
3. **Bayer, M. G., J. H. Heinrichs, and A. L. Cheung.** 1996. The molecular architecture of the *sar* locus in *Staphylococcus aureus*. J. Bacteriol. **178:**4563–4570.
4. **Byrne, M. E., D. A. Rouch, and R. A. Skurray.** 1989. Nucleotide sequence analysis of IS*256* from the *Staphylococcus aureus* gentamicin-tobramycin-kanamycin-resistance transposon Tn*4001*. Gene **81:**361–367.
5. **Chambers, H. F.** 1997. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. Clin. Microbiol. Rev. **10:**781–791.
6. **Couto, I., H. de Lencastre, E. Severina, W. E. Kloos, J. A. Webster, R. J. Hubner, I. Santos Sanches, and A. Tomasz.** 1996. Methicillin resistance in *Staphylococcus sciuri*. Microb. Drug Resist. **2:**377–391.
7. **Couto, I., I. Santos Sanches, R. Sá-Leão, and H. de Lencastre.** 2000. Molecular characterization of *Staphylococcus sciuri* strains isolated from humans. J. Clin. Microbiol. **38:**1136–1143.
8. **Dyke, K. G., S. Aubert, and N. el Solh.** 1992. Multiple copies of IS*256* in staphylococci. Plasmid **28:**235–246.
9. **Firth, N., and R. A. Skurray.** 2000. The *Staphylococcus* genetics: accessory elements and genetic exchange, p. 326–338. *In* V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy, and J. I. Rood (ed.), Gram-positive pathogens. ASM Press, Washington, D.C.
10. **Hartman, B. J., and A. Tomasz.** 1984. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus.* J. Bacteriol. **158:**513–516.
11. **Hedin, G., and M. Widerström.** 1998. Endocarditis due to *Staphylococcus sciuri*. Eur. J. Clin. Microbiol. Infect. Dis. **17:**673–674.
12. **Kloos, W. E., D. N. Ballard, J. A. Webster, R. J. Hubner, A. Tomasz, I. Couto, G. Sloan, H. P. Dehart, F. Fiedler, K. Schubert, H. de Lencastre, I. Santos Sanches, H. E. Heath, P. A. Leblanc, and A. Ljungh.** 1997. Ribotype delineation and description of *Staphylococcus sciuri* subspecies and their potential as reservoirs of methicillin resistance and staphylolytic enzyme genes. Int. J. Syst. Bacteriol. **47:**313–323.
13. **Kolawole, D. O., and A. O. Shittu.** 1997. Unusual recovery of animal staphylococci from septic wounds of hospital patients in Ile-Ife, Nigeria. Lett. Appl. Microbiol. **24:**87–90.
14. **Lyon, B. R., M. T. Gillespie, and R. A. Skurray.** 1987. Detection and characterization of IS*256*, an insertion sequence in *Staphylococcus aureus*. J. Gen. Microbiol. **133:**3031–3038.
15. **Maki, H., and K. Murakami.** 1997. Formation of potent hybrid promoters of the mutant *llm* gene by IS*256* transposition in methicillin-resistant *Staphylococcus aureus*. J. Bacteriol. **179:**6944–6948.
16. **Marsou, R., M. Bes, M. Boudouma, Y. Brun, H. Meugnier, J. Freney, F. Vandenesch, and J. Etienne.** 1999. Distribution of *Staphylococcus sciuri* subspecies among human clinical specimens, and profile of antibiotic resistance. Res. Microbiol. **150:**531–541.
17. **Matthews, P., and A. Tomasz.** 1990. Insertional inactivation of the *mec* gene in a transposon mutant of a methicillin-resistant clinical isolate of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **34:**1777–1779.
18. **Picardeau, M., A. Varnerot, J. Rauzier, B. Gicquel, and V. Vincent.** 1996. *Mycobacterium xenopi* IS*1395*, a novel insertion sequence expanding the IS*256* family. Microbiology **142:**2453–2461.
19. **Rice, L. B., and A. S. Thorisdottir.** 1994. The prevalence of sequences homologous to IS*256* in clinical enterococcal isolates. Plasmid **32:**344–349.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Schleifer, K. H., and R. M. Kroppenstedt.** 1990. Chemical and molecular classification of staphylococci. J. Appl. Bacteriol. Symp. Suppl. **69:**9S–24S.
22. **Servin-Massieu, M.** 1961. Spontaneous appearance of sectored colonies in *Staphylococcus aureus* cultures. J. Bacteriol. **82:**316–317.
23. **Sieradzki, K., M. G. Pinho, and A. Tomasz.** 1999. Inactivated *pbp4* in highly glycopeptide-resistant laboratory mutants of *Staphylococcus aureus.* J. Biol. Chem. **274:**18942–18946.
24. **Takahashi, T., I. Satoh, and N. Kikuchi.** 1999. Phylogenetic relationships of 38 taxa of the genus *Staphylococcus* based on 16S rRNA gene sequence analysis. Int. J. Syst. Bacteriol. **49:**725–728.
25. **Udo, E. E., L. E. Jacob, and T. D. Chugh.** 1995. Antimicrobial resistance of coagulase-negative staphylococci from a Kuwait hospital. Microb. Drug Resist. **1:**315–320.
26. **Wallet, F., L. Stuit, E. Boulanger, M. Roussel-Delvallez, P. Dequiedt, and R. J. Courcol.** 2000. Peritonitis due to *Staphylococcus sciuri* in a patient on continuous ambulatory peritoneal dialysis. Scand. J. Infect. Dis. **32:**697–698.
27. **Wu, S., C. Piscitelli, H. de Lencastre, and A. Tomasz.** 1996. Tracking the evolutionary origin of the methicillin resistance gene: cloning and sequencing of a homologue of *mecA* from a methicillin susceptible strain of *Staphylococcus sciuri*. Microb. Drug Resist. **2:**435–441.
28. **Wu, S., H. de Lencastre, and A. Tomasz.** 1998. Genetic organization of the *mecA* region in methicillin-susceptible and methicillin-resistant strains of *Staphylococcus sciuri*. J. Bacteriol. **180:**236–242.
29. **Wu, S. W., H. de Lencastre, and A. Tomasz.** 2001. Recruitment of the *mecA* gene homologue of *Staphylococcus sciuri* into a resistance determinant and expression of the resistant phenotype in *Staphylococcus aureus*. J. Bacteriol. **183:**2417–2424.
30. **Ziebuhr, W., V. Krimmer, S. Rachid, I. Lossner, F. Gotz, and J. Hacker.** 1999. A novel mechanism of phase variation of virulence in *Staphylococcus epidermidis*: evidence for control of the polysaccharide intercellular adhesin synthesis by alternating insertion and excision of the insertion sequence element IS*256*. Mol. Microbiol. **32:**345–356.